Minireview

Probing membrane protein properties using droplet interface bilayers

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Impact statement

The paper presents a comprehensive review of integral membrane protein studies utilizing droplet interface bilayers. Droplet interface bilayers are a novel method of constructing artificial lipid bilayers with enhanced stability and physicochemical complexity compared to existing methods. Their unique morphology also suggests applications in the construction of synthetic biological systems and protocells. As well as serving as a guide to *in vitro* membrane protein functional studies using droplet interface bilayers in the literature to date, a novel *in vitro* study of a flippase protein in a droplet interface bilayer is presented.

Abstract

Integral membrane proteins comprise a large proportion of drug targets, yet are challenging to study *in vitro* due to their amphiphilic nature. Conducting useful functional *in vitro* studies requires an artificial membrane that can mimic the lipid environment of the biogenic membrane. Droplet interface bilayer technology provides a method to form artificial bilayers with a robustness and physicochemical complexity that has not previously been possible, facilitating more sophisticated *in vitro* studies of membrane proteins. This mini-review examines functional studies of membrane proteins that utilize droplet interface bilayers to date and comments on possible directions of future research. Observations from our own laboratory regarding the study of a flippase protein in droplet interface bilayers are also presented.

Keywords: Membrane proteins, droplet interface bilayer, droplet interface bilayer, synthetic biology, artificial bilayer, flippase

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Introduction

One of the great challenges when studying proteins *in vitro* is creating an environment approximating that found *in vivo*. The cellular environment comprises a vastly complex interacting network of biomolecules across length scales from microns to nanometers. Cells are bounded by equally complex membranes comprised of an asymmetric lipid bilayer with many associated proteins, both peripherally and spanning the bilayer as integral membrane proteins.[1] Integral membrane proteins are involved in a diverse array of cellular functions, including intracellular signaling and regulating the influx and efflux of molecules to the cell.[2] They are one of the major classes of drug target and as such are an attractive and important subject of study.[3]

Studying integral membrane proteins *in vitro* presents an additional set of challenges over soluble proteins. Membrane proteins are amphiphilic, featuring a hydrophobic bilayer-spanning transmembrane region. In order to study them in an environment resembling the biogenic membrane, an artificial lipid bilayer must first be created and the protein reconstituted into the membrane.

Liposomes, small vesicles bounded by a lipid bilayer, are well established as a platform for mimicking the biogenic membrane with an extensive history of use in functional studies of membrane proteins.[4] Liposomes are a versatile host for membrane proteins, but their closed nature allows access to only one side of the bilayer and little control over protein orientation is possible. This can complicate electrophysiological and fluorescent measurements of membrane protein activity.[5,6] Planar bilayers can overcome these challenges by permitting the experimenter access to both sides of the lipid bilayer.

The earliest techniques developed for constructing planar artificial lipid membranes involved creating a free-standing lipid membrane spanning an aperture between two compartments filled with an aqueous solution.[2] This can be accomplished by painting a solution of lipid in an organic solvent over a small aperture in a Teflon partition between aqueous reservoirs.[7] This mixture forms a thin film over the aperture which spontaneously thins to

eventually form a lipid bilayer, known as a black lipid bilayer or aperture-suspended lipid bilayer. Alternatively, the Teflon partition can be lowered slowly into an aqueous bath with a self-assembled lipid monolayer on its surface, which subsequently forms a solvent-free bilayer spanning the aperture, known as a Montal Mueller lipid bilayer.[8]

Black lipid and Montal Mueller bilayers are mechanically unstable structures, with half lives of a few hours, making them a difficult platform upon which to study membrane protein function.[9] Other techniques are available for generating lipid bilayers supported on a surface, such as fusing lipid vesicles with a supported lipid monolayer.[10] Supported bilayers only leave one side of the membrane accessible and often suffer from surface defects and problems of non-uniformity.[2]

The droplet interface bilayer

An ideal artificial bilayer for studying membrane protein function *in vitro* should be easy to construct, mechanically stable and present the bilayer in such a way that both sides can be accessed. The droplet interface bilayer (DIB), a relatively recently devised method for forming artificial bilayers, fulfills all of these requirements.[9,11] DIBs are formed from the contact of two aqueous droplets coated with a lipid monolayer in a non-aqueous "oil" bath (Figure 1). Small (nanoliter) volume aqueous droplets are

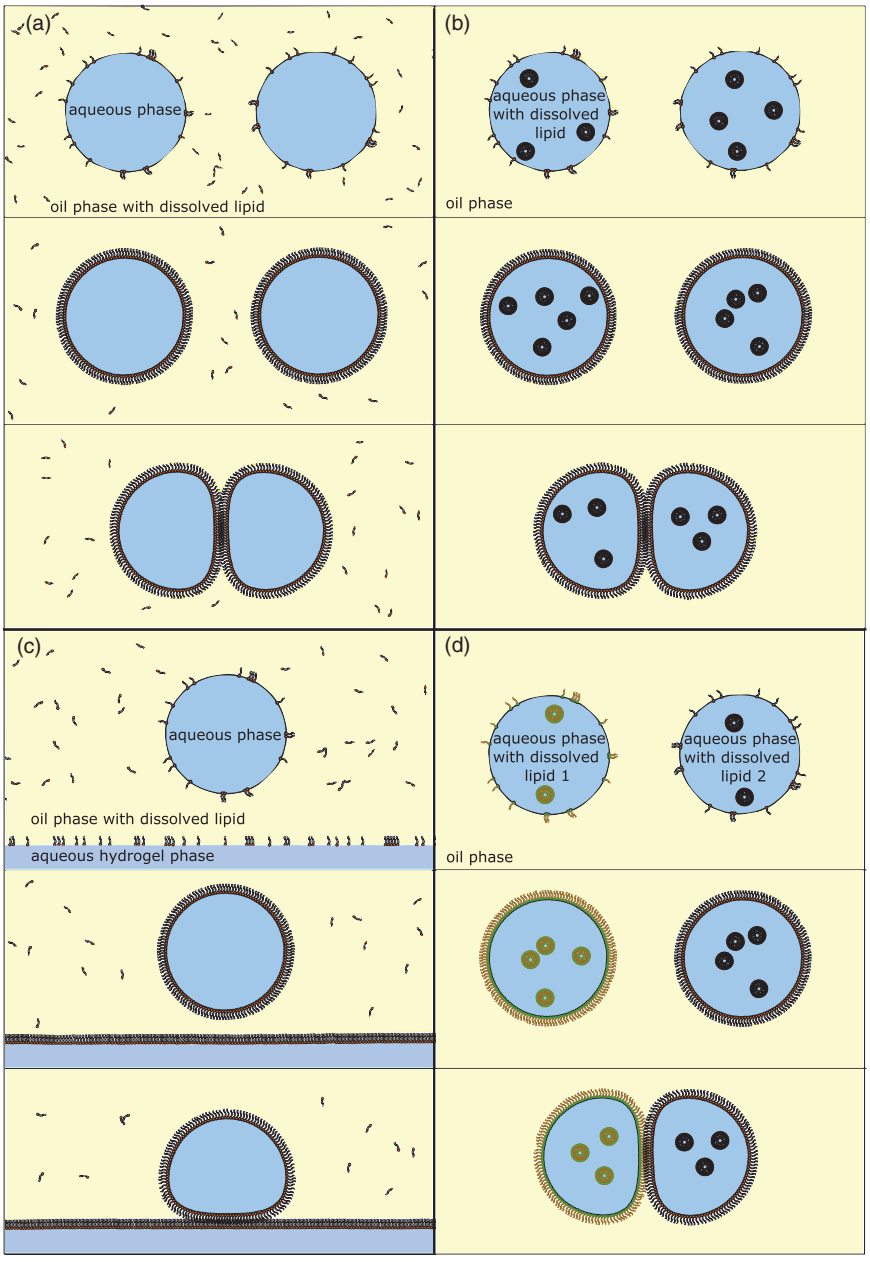

Figure 1. Schematic diagram of the assembly of a droplet interface bilayer using a variety of techniques. Panel a: the "lipid in" technique, panel b: the "lipid out" technique, panel c: the "lipid out" droplet hydrogel bilayer, panel d: the "lipid in" asymmetric bilayer. The droplets are shown in their initial state in the top section of each panel; monolayer formation is shown in the middle section and bilayer formation in the bottom section. (A color version of this figure is available in the online journal.)

pipetted into an oil bath, usually a hydrocarbon such as hexadecane. Lipid is supplied to the system either as liposomes in the aqueous droplets ("lipid-in") or dissolved the oil ("lipid-out").[9] The droplets are then incubated to allow a self-assembled monolayer of lipid to form on the surface of the droplet. The incubation time varies from around 5 min for a "lipid-in" DIB to around 30 for a "lipid-out" DIB due to the differing kinetic of monolayer formation in the two cases.[12] Once a monolayer is formed, the droplets are moved gently together manually or using micromanipulators. On contact, the oil between them is displaced and a planar lipid bilayer is formed.

DIBs produced this way are very stable, with half-lives ranging from hours to days.[9] This stability makes them an excellent platform for the functional study of membrane proteins. DIBs can be used to perform electrophysiological characterization of membrane channels by forming the droplets on the tip of agarose-coated electrodes.[9,13,14] This also permits easy spatial manipulation of the droplets in the oil bath using micromanipulators. By moving the droplets towards or away from each other, the precise size of the bilayer can be controlled.[13] Moving the droplets even further apart allows the bilayer to be broken and reformed without rupture.[15,16]

DIBs are not restricted to a single interface between two droplets. Interfaces can be formed between many droplets, creating networks of compartments bounded by lipid bilayers.[17] A morphology similar to that of a supported bilayer can also be created by forming an interface between a single droplet and a lipid monolayer assembled on a planar hydrogel support, known as a droplet hydrogel bilayer.[18,19] This arrangement permits the use of TIRF microscopy to illuminate the bilayer. Microfluidic devices have also been used to rapidly and precisely form DIBs.[11,20,21]

DIB networks, droplet hydrogel bilayers, and microfluidically formed DIBs have been reviewed extensively elsewhere.[2,9,17,22–24] This review will concern itself mainly with DIBs composed of two droplets as a tool for studying membrane protein properties. Reconstituting membrane proteins into DIBs and exploiting the unique properties of DIBs in functional studies will be covered, as well as potential difficulties that may be encountered. Some observations from our own laboratory regarding reconstituting and characterizing a lipid flippase in a DIB will also be discussed.

Incorporating proteins into DIBs

Spontaneous insertion from solution

The methods used to incorporate a protein into a DIB depend both on the protein itself and on the purpose of the study. The first protein to be incorporated into a DIB was the pore-forming toxin α-hemolysin (αHL).[9,11,13] Monomeric αHL is water soluble and binds to lipid membranes, oligermerizing into a heptameric, β-barrel water-permeable pore.[25] As such, incorporating αHL into a DIB is relatively trivial as it will spontaneously insert into a DIB membrane and form pores directly from solution.[13] This has led to αHL being widely used to selectively permeabilize membranes in DIB networks with a view to altering their electrical properties.[15,26–29] Aside from studies characterizing αHL pore properties by either electrical[9] or optical means,[24] αHL more often finds use in DIBs as a tool to alter the properties of the bilayer rather than an object of study itself. The use of αHL as a tool to selectively permeabilize membranes with a pore of known morphology has become quite widespread, finding application in such fields as the construction of artificial cellular systems and single molecule sensing.[30–33]

Spontaneous insertion from detergent micelles and liposomes

Ion channels and transporters, which may be more intriguing subjects for characterization using DIBs, do not typically have a water soluble form such as the αHL monomer.[25] Purifying integral membrane proteins for *in vitro* study requires an amphiphile such as a detergent micelle or a lipid nanostructure (e.g. liposomes or bicelles) to solubilize the protein.[34] Incorporating most membrane proteins into a DIB typically begins with a purified protein sample solubilized in either detergent micelles or liposomes which must then be functionally reconstituted into a DIB membrane. A summary of proteins incorporated to date into DIBs can be found in Table 1.

Some proteins can insert into DIBs directly from detergent micelles. The light-driven proton pump bacteriorhodopsin (BR) is known to spontaneously insert into a DIB membrane directly from detergent in a functional form, without the need for any additional preparation.[9,13,41] The bacterial outer membrane porin OmpG has also been inserted directly into a DIB from detergent micelles, and subsequently electrically characterized.[9,14] This did not occur spontaneously, however requiring an applied potential of 100 mV, in a similar manner to the potential required to insert OmpG into planar bilayers.[45]

The reconstitution method which has proved perhaps the most adaptable in incorporating a wide variety of proteins into DIBs is spontaneous insertion from liposomes. The potassium channels Kv1.1, KcsA, and Kcv, the mechanosensitive channel MscL, and the disaccharide transporter LacY have all been incorporated into DIBs using this method.[9,15,36,40,44] In this method, liposomes are swollen by mixing with a detergent such as octyl glucoside (OG) before mixing with detergent solubilized protein. Excess detergent is then removed, often using adsorbent beads such as BioBeads, leaving proteoliposomes containing the protein of interest. These are then used as the lipid source for a "lipid-in" DIB, with the proteins becoming incorporated into the interface.[40,44]

This incorporation method, though versatile, is not without its drawbacks. The process by which membrane proteins exchange into a DIB from proteoliposomes is not well understood, leaving it difficult to ascertain exactly how much protein is present in the DIB interface and how much remains within proteoliposomes in the droplet. As such, any quantified transport rates can only represent a lower bound of measured activity, if it is assumed that all of the protein is incorporated into the interface. An

Table 1. Membrane proteins successfully reconstituted into DIBs to date and the method of reconstitution employed.

Protein	Class	Method of reconstitution into a DIB
αhl	Pore forming toxin from *Staphylococcus aureus*	Spontaneous insertion from solution[9,13,14,24,26–28,] and direct insertion from IVTT[15,29,35]
OmpG	Outer membrane pore from *Escherichia coli*	Insertion from detergent micelles under an applied potential[9,14]
Kcv	Potassium channel from PBCV-1 chlorella virus	Insertion from solution under an applied potential[9] and direct insertion from IVTT[15]
Kv1.1 and Kv1.3	Human potassium channel	Spontaneous insertion from liposomes[36]
KcsA	Potassium channel *from Streptomyces lividans*	Spontaneous insertion from solution with a transmembrane electrochemical gradient,[9,37] incorporation from cell membrane fragments[38] and insertion from IVTT[39]
MscL	Mechanosensitive channel from *Escherichia coli*	Spontaneous insertion from liposomes[40]
Bacteriorhodopsin (BR)	Archael light-driven proton pump	Spontaneous insertion from detergent micelles[9,13,41]
MmPiezo and DmPiezo	Mechanosensitive proteins from mouse (MmPiezo) and *Drosophila melanogaster* (DmPiezo)	Spontaneous insertion from liposomes[42]
Wza	Polysaccharide exporter from *Escherichia coli*	Spontaneous insertion from liposomes[43]
LacY	β-galactoside synporter from *Escherichia coli*	Spontaneous insertion from liposomes and insertion from IVTT[44]
NMDA receptor	Glutamate receptor and ion channel	Incorporation from cell membrane fragments[38]
hERG	Human potassium channel	Incorporation from cell membrane fragments[38] and insertion from IVTT[39]

DIB: droplet interface bilayer. IVTT: *in vitro* transcription translation

improved understanding of the mechanism and kinetics of the exchange of proteins into the DIB would permit a more detailed measure of protein activity to be gleaned from studies employing this insertion method.

Insertion from cell membrane fragments

Protein insertion into droplet hydrogel bilayers has been successfully accomplished directly from cell membrane fragments, negating the need for solubilizing and reconstituting membrane proteins.[38] Leptihn *et al.* applied a solution of isolated cell membrane fragments from a porin-free *Escherichia coli* (*E. coli*) strain overexpressing the potassium channel KcsA to an agarose layer on a glass cover slip, followed by a solution of phospholipids in hexadecane. This formed a lipid monolayer over the agarose surface. An aqueous droplet was subsequently introduced, acquiring its own monolayer coating and forming a droplet-hydrogel bilayer upon contact with the agarose supported monolayer (Figure 2). Electrical characterization via electrodes in the droplet and embedded in the agarose layer revealed conductance properties characteristic of single KcsA channels. The experiment was repeated using membrane fragments from cells overexpressing the hERG channel, mouse fibroblast cells overexpressing human NMDA receptors and SupT1 lymphocyte cells. Single channel current measurements were recorded for both the hERG channels and NMDA receptors. This is a promising method for incorporating proteins too fragile to survive purification and solubilization into artificial lipid bilayers, and can be applied to a variety of cells, both prokaryotic and eukaryotic. This technique is however limited, at present, to inserting proteins into droplet-hydrogel bilayers.

Insertion from IVTT

Another strategy for incorporating proteins into DIBs that avoids the protein purification process is direct insertion from *in vitro* transcription and translation (IVTT) systems. IVTT, also referred to as cell-free protein synthesis, is a method for producing protein directly from extracted cellular transcription and translation machinery.[46] Cell-free protein synthesis initially relied on extracts of cell lysate but has progressed to using individual purified transcription and translation complexes supplemented with small molecules, such as the PURExpress®.[46–48] Protein synthesis is accomplished simply by incubating DNA encoding the protein of interest with the cell-free synthesis cocktail. Localizing a cell-free synthesis system to one droplet of a DIB interface allows a protein to be synthesized and its activity interrogated *in situ*. This is a particularly attractive technique for producing fragile or toxic transporters and channels for which largescale overexpression and purification may not be practical.

Cell-free protein synthesis of membrane proteins in a DIB has been successfully demonstrated using αHL,[15,29,41] Kcv,[15] LacY,[44] KcsA, and the hERG pore domain.[39] Usually an IVTT reaction is initiated in bulk and the mixture subsequently used to form DIBs. However, Booth *et al.*[29] have demonstrated the cell free synthesis of αHL in a DIB network after bilayer formation by employing a light sensitive DNA promoter to control the initiation of protein synthesis.

Syeda et al. produced both Kcv and αHL using an IVTT system subsequently used to form a DIB and characterized the electrical properties of the channels.[15] They also used the fact that DIBs can be broken and reformed to construct a droplet array system, consisting of a number of fixed half-droplets and a moveable droplet containing Kcv, to screen the activity of a number of channel inhibitors.

The authors also conducted a comparative study of different cell-free synthesis systems, comparing the effectiveness of an extract-based system (the Promega S30 extract) with the PURExpress® system. The authors note that DIBS made from droplets containing the PURExpress® system were significantly more stable, lasting an average of 8.7 h, when compared to the S30 extract, which only lasted on average 0.7 h. The proteins produced by both systems

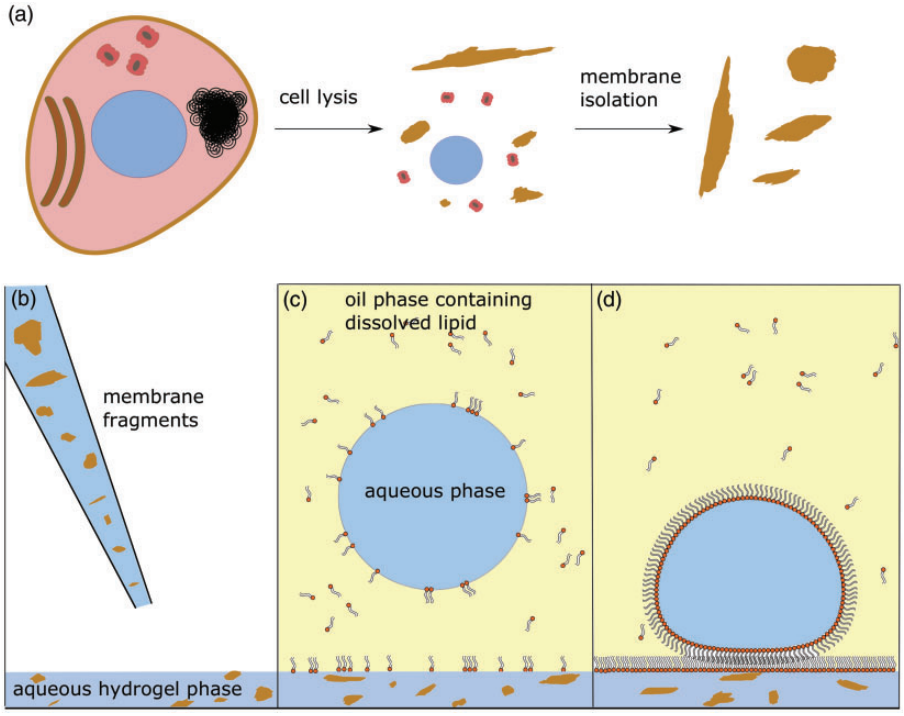

Figure 2. Schematic diagram showing the formation of a droplet hydrogel bilayer incorporating cell membrane fragments. Panel a: cells are lysed and the membrane fraction separated. Panel b: cell membrane fragments are pipetted onto a hydrogel coated coverslip. Panels c and d: a droplet hydrogel bilayer is formed atop the hydrogel layer with incorporated membrane fragments. (A color version of this figure is available in the online journal.)

showed comparable electrical properties, but the PURExpress® droplets required pre-incubation at 37°C to produce protein, whereas those with the S30 extract produced protein at room temperature. This is likely a consequence of the PURExpress® system being optimized for protein expression at 37°C.[47,48] Friddin *et al*. have investigated the effects of a number of commercial lysate-based IVTT systems on the stability of DIBs.[49] As well as identifying a number of components of the systems detrimental to the integrity of the bilayer, the authors demonstrate that the addition of lipid vesicles to the system can significantly improve the overall stability of the DIB.

Cell-free protein synthesis in DIBs has some shortcomings. As stated earlier, a pre-incubation period at 37°C is required when using the PURExpress® system, which may necessitate the use of a temperature controlled oil bath if protein synthesis must be initiated after DIB formation. Cell-free protein synthesis also places constraints on the buffer composition of the synthesizing droplet, as the PURExpress® system is buffered to maximize protein production.[48] Syeda *et al*. note that it is possible to alter the buffer conditions somewhat without overly compromising protein synthesis, adding 250 mM of KCl and obtaining sufficient protein for their experiments.[15] This suggests that there may be some leeway in buffer conditions which produce acceptable protein synthesis in the PURExpress® system.

Bilayer composition

A study of membrane protein function employing a DIB, or indeed any bilayer, should give due consideration to the chemical identity of the lipids employed. Bilayer charge, thickness, lateral pressure, and headgroup chemical composition all influence membrane protein folding, stability, and function.[50] In addition to forming a mechanically robust bilayer, the protein must be stabilized in an active state. Many early DIB studies were conducted using the lipid diphytanoylphosphatidylcholine (DPhPC).[9,13,26] DPhPC forms fluid bilayers with excellent temperature and mechanical stability and high electrical resistance.[51,52] These attributes are desirable when performing electrophysiological measurements and when forming large networks of DIBs.[9] Taken with the propensity of αHL to insert easily into DPhPC DIBs, it still finds widespread use in functionalized DIB networks.[17,29,35]

DPhPC has some less desirable properties which can make it a problematic lipid to use for studies of ion channels and transporters. It is a synthetic lipid, with branched acyl chains that more closely resemble lipids found in archaea than other prokaryotes and eukaryotes.[52] This can cause problems when designing a lipid environment for studying bacterial or eukaryotic proteins, as the resulting membrane will bear little physicochemical resemblance to the biogenic lipid environment.

Renauld *et al*. attempted to electrically characterize the potassium channel Kv1.1 produced via cell-free protein synthesis using a DIB composed of DPhPC.[36] They observed notably higher conductance values for the channel compared to previously published studies, along with the loss of voltage gating behavior. The authors speculate that this behavior was due to a loss of associated lipid derived function that could not be replicated in the synthetic DPhPC bilayer. They attribute the loss of voltage gating behavior to the zwitterionic nature of DPhPC, with charge coupling

to a cationic lipid via the S4 helix being a pre-requisite for voltage gating.[53] This demonstrates that a simple DPhPC bilayer, while being a convenient platform for DIB formation, may be inadequate as a replica of the biogenic membrane for membrane protein functional studies.

One early study utilizing lipids other than DPhPC to form DIBs was carried out by Aghdaei *et al*.[37] The team utilized asolectin, 1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1′-rac-glycerol) (POPG) in addition to DPhPC to form bilayers. Rather than bringing lipid-coated droplets together manually to form DIBs, the team used an innovative dielectrophoresis system to manipulate them. As well as characterizing the bilayer electrically, the team also reconstituted the ion channel KcsA into the bilayer and were able to record single channel conductance measurements.

A subsequent study characterizing a membrane protein in a DIB made from a lipid other than DPhPC was carried out by Barriga *et al*.[40] The purified *E. coli* mechanosensitive channel was reconstituted into a DIB formed from 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and its gating properties, in the presence of a chemical gating agent, characterized using fluorescence techniques.

More recently, Findlay *et al*. reconstituted the disaccharide transporter LacY into DIBs constructed of a mixture of three different lipids, via both spontaneous insertion from purified protein in liposomes and directly from cell-free protein synthesis.[44] By using a mixture of bilayer forming DOPC, non-lamellar 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and anionic 1,2-dioleoyl-sn-glycero-3-phosph-(1′-rac-glycerol) (DOPG) lipids, the lateral pressure and charge of the bilayer could be altered. Using a fluorescent lactose analogue, the team characterized the effects of varying these bilayer properties on protein activity, recording the highest transport activity when all three lipids were present in a 4:4:2 ratio (Figure 3). LacY is endogenous to the *E. coli* inner cytoplasmic membrane, which is composed primarily of phosphatidylethanolamine lipids (approx 75%), along with anionic phosphatidylglycerol lipids (approx. 20%) and cardiolipin.[54] A DIB containing a significant proportion of non-bilayer forming and charged lipids might thus be expected to promote increased transport activity over a membrane lacking these properties.

Studies such as these demonstrate the potential of DIBs as a platform to study the interplay between membrane proteins and lipids. Aside from the studies discussed, there has been relatively little work on expanding the range of biological membranes that can be mimicked as DIBs. One recent study of note carried out by Barlow *et al*.[55] constructed a number of model DIB membranes mimicking the lipid composition of several plants. Further developments in this field will be needed for the full potential of DIBs as an *in vitro* lipid membrane model system to be realized.

Unique properties of DIBs

DIBs possess a number of unique properties not found in other planar lipid bilayer models or lipid nanostructures such as liposomes and nanodiscs. As well as being significantly more stable than black lipid and Montal Mueller bilayers, DIBs can be made with far smaller aqueous volumes.[9] Forming asymmetric lipid bilayers using DIBs is also relatively trivial. The following section will examine a number of studies that have exploited the unique properties of small volumes and asymmetric bilayers to study membrane proteins in a manner impossible in other model membranes.

Small volume

Black lipid and Montal Mueller lipid bilayers typically use aqueous chambers closer to milliliters in size, with more novel microfabrication methods able to reduce this to

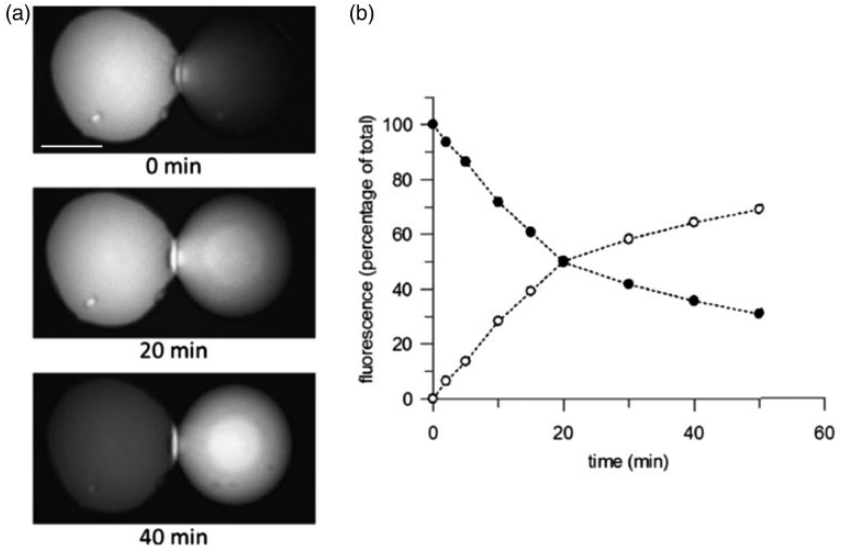

Figure 3. Transport of a fluorescent lactose analogue across a 40:40:20 (mole ratio) DOPC:DOPE:DOPG DIB by LacY against a concentration gradient. Panel a: fluorescent images of the DIB at various time points. Scale bar is 500 μm. Panel b: relative fluorescence as a percentage of total fluorescence over time of the left (closed circle) and right (open circle) droplets. Reproduced with permission from Findlay *et al*.[44]

microliters.[2] DIBs can be formed from droplets ranging in size from hundreds of nanoliters down to femtoliters,[35,56] orders of magnitude smaller than black lipid and Montal Mueller bilayers. This corresponds to an increase in the concentration of reagents and substrates that can be achieved within the droplets. Higher reagent concentrations within droplets speeds up reactions, permits the use of rare reagents at high concentrations, and greatly enhances the sensitivity of measurements of protein activity using optical or electrical methods.[2] A small volume is also a factor in enabling effective cell-free protein synthesis in DIBs in quantities sufficient for functional studies.[44]

A study by Kong *et al*. demonstrates how the very small volumes attainable through DIBs facilitate studies that would be impossible in larger bilayer systems.[43] First, fragments of various lengths of the *E. coli* K30 capsular polysaccharide were synthesized using a novel method of multi-step polyglycosylation. The interaction between these fragments and the bacterial sugar exporter protein Wza was then characterized electrically across a DIB. Using small droplets (around 200 nL), high concentrations of polysaccharide (>10 mM) could be attained from very small amounts of synthesized material. This greatly increased the probability of detecting binding events between the K30 fragments and the Wza pore.

A number of studies have examined the morphologies and stabilities of DIBs in the very small (femtoliter) volume range.[56,57] The authors in both studies find DIBs of this size to be fundamentally unstable, with evaporative effects causing dynamic morphological changes depending on the initial conditions of the system, leading to eventual destruction of the bilayer. Guiselin *et al*. provide a thorough theoretical treatment of the stability and kinetics of DIBs in this size range.[58]

Bilayer asymmetry

Creating an asymmetric lipid bilayer, with a differing lipid composition in each leaflet, is a challenging prospect using conventional bilayer formation techniques but can be accomplished relatively easily in DIBs.[9,14] Using the "lipid-in" technique, a DIB can be formed from two droplets of liposomes of differing lipid compositions, leading to an asymmetric membrane at the interface. Most biogenic membranes are asymmetric, and the *in vitro* study of the effects of asymmetry on membrane protein function has, to date, been limited.

Hwang *et al*. were the first to employ an asymmetric DIB to characterize protein function in a study on the effects of membrane surface charge asymmetry on the gating of the *E. coli* porin OmpG.[14] The authors initially electrically characterized the insertion from detergent micelles and gating behavior of OmpG in a symmetrical DPhPC bilayer under an applied potential, finding equivalent results to those found in planar bilayers. The experiment was then repeated with an asymmetric bilayer, one leaflet supplemented with 10 mol% of a negatively charged lipid (1,2-dipalmitoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] (DPPG)) and the other 10 mol% of a positively charged lipid (dimethyldioctadecylammonium bromide). When inserted from the negative side of the membrane, OmpG was found to have a much increased frequency of gating compared to a neutral bilayer, and a much decreased gating frequency when inserted from the positive side of the membrane. The authors speculate that the difference in gating behavior was derived from interactions between negatively charged extracellular loops and the charged membrane.

The authors also performed an experiment to confirm that asymmetric DIBs did maintain their asymmetry over the course of the experiment. Using fluorescent lipids, they were able to measure the background rate of intra-leaflet lipid movement, finding that its half-life was on the order of hours to days. This confirmed that asymmetry could be maintained across a DIB over the timescale required to conduct measurements. Electrophysiological measurements across asymmetric DIBs have been employed by other groups, notably by Coste *et al.* who investigated the channel properties of a number of mechanically sensitive proteins.[42] More recently, Taylor *et al*. have interrogated the stability of asymmetric bilayers using parallel electrophysiological recordings across an array of DIBs.[59] As well as confirming the stability of asymmetric lipid compositions over experimental timescales, the authors find that the addition of the peptide alamethicin can promote intra-leaflet lipid flipping in its surface bound state.

A recent series of studies used asymmetric DIBs to characterize the effect of membrane potential on Pep-1 peptide mediate protein transport without applying an external potential to the membrane.[60,61] Pep-1 is a membrane transport peptide which forms non-covalent, membrane permeable complexes with proteins, though the exact mechanism of how this occurs is unclear.[62] An initial study used asymmetric DIBs, supplemented with negatively charged lipids in one leaflet, to characterize the movement of Pep-1 bound to horseradish peroxidase (HRP) across asymmetrically charged bilayer.[60] HRP was employed to catalyze a fluorescent assay reaction in the second drop, allowing Pep-1-mediated transport across the membrane to be quantified. Pep-1-mediated HRP transport was only observed when negative charge was present on the opposing membrane from the droplet containing Pep-1 and HRP. A subsequent study replicated these results, as well as characterizing the effects of altering the Pep-1 to HRP ratio and investigating the effect of headgroup variation of the non-charged lipids on transport.[61]

Aside from a few exceptions, the use of DIBs to study the role that lipid membrane asymmetry has on protein function is currently not very widespread. Considering the ubiquity of asymmetry in biogenic lipid membranes, DIBs have great potential to become a key tool to enable studies that examine the effects of membrane asymmetry on proteins *in vitro*.

MsbA

Recently in our laboratory, we undertook an investigation using asymmetric DIBs to characterize a lipid flippase. Lipid flippases are proteins which catalyze the intra-bilayer movement of lipids from one leaflet to the other.[63] Flippase proteins are required to generate and maintain

membrane asymmetry *in vivo* and to modulate it during cellular process, such as apoptosis, that involve transbilayer lipid movement. DIBs are an attractive artificial membrane platform for studying flippases *in vitro* due to the easy of forming asymmetric membrane and of monitoring the transbilayer movement of fluorescently labeled lipids.

MsbA is an ATP-binding cassette type transporter from *E. coli*, implicated in translocating the lipid component of lipopolysaccharide, lipid A, across the cytoplasmic membrane.[64] It is a relatively well-characterized protein, with its lipid A bound structure recently having been solved using cryo electron microscopy.[65] Functional studies have also demonstrated that its specificity is broad, extending to a range of lipids and hydrophobic molecules, and that it retains flippase activity in vesicles.[66–68]

These attributes make MsbA an ideal test protein for assessing DIBs as a platform for studying protein-mediated lipid flipping. Eckford *et al.* have shown that MsbA will flip phosphatidylethanolamine (PE) lipids functionalized with the fluorescent label 7-nitrobenz-2-oxa-1,3-diazole (NBD) in vesicles.[68] We therefore decided to create an asymmetric DIB labeled with NBD-PE lipids present on one side with MsbA reconstituted into the interface. Movement of the fluorescently labeled lipids from one leaflet to the other could then be measured by measuring the fluorescent intensity of the two droplets.

The extent of the effect that the lipid environment had on the activity of MsbA was investigated. MsbA was recombinantly overexpressed and purified as described in the supplementary materials (S1) and reconstituted into liposomes of various lipid compositions. ATPase activity (as a proxy for flippase activity) was measured using a commercially available linked assay (Invitrogen^{TM} EnzChek^{TM} Phosphate Assay Kit). The highest reconstitution efficiencies and ATPase activities were seen in a mixture of DOPC, DOPG, and DOPE, in an *E. coli* polar lipid extract and in 95% DPhPC supplemented with 5% NBD labeled PE (Figure 4).

Further study involved assessing translocation of lipids by reconstituted MsbA from one monolayer leaflet to the other, with this protein-mediated lipid flipping potentially causing mechanical disruption to the DIB. DPhPC provides particularly robust DIBs and thus a bilayer composed primarily of DPhPC was chosen for the initial studies presented here. However, membrane proteins often exhibit low activity in DPhPC, as found here. The addition of small proportions of PE lipids to PC bilayers has been shown, under certain buffer conditions, to induce particularly high levels of membrane protein activity, greater than that induced by higher proportions of PE lipids.[69] We therefore included 5% NBD-labeled PE in the DPhPC bilayer for visualization of the lipid movement on the microscope as well as to promote protein activity.

To form an asymmetric DIB, purified MsbA was first reconstituted into liposomes formed of 95% DPhPC and 5% NBD-labeled DOPE by mass. The liposomes were then diluted to 5 mg/mL of lipid in a buffer containing ATP. A 400 nL droplet of this mixture was then pipetted into a hexadecane bath to form one droplet (the "donor" droplet) of the asymmetric DIB. A 400 nL droplet of 5 mg/mL DPhPC liposomes in identical buffer was then pipetted

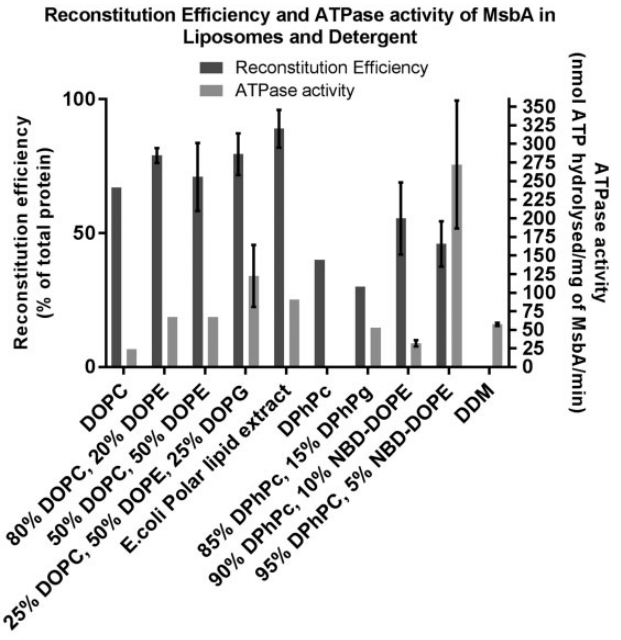

Figure 4. Graph showing reconstitution efficiency and ATPase activity of MsbA reconstituted into liposomes of differing lipid compositions and of detergent.

into the bath (the "acceptor" droplet), and the two droplets drought together after a 5 minute incubation period to form an asymmetric "lipid in" DIB.

The droplets were then monitored on a fluorescence microscope over 11 hours. Figure 5 shows the droplets at $t = 0$ (panel a) and $t = 500$ minutes (panel b). Panel c shows the distribution of fluorescence between the two droplets over time. In order to account for the uniform effect of photobleaching over time, the fluorescence of the "acceptor" droplet is shown as a percentage of the sum total fluorescence of both droplets for each time point. The percentage of the total fluorescence of the two droplets that was localized in the "acceptor" droplet grew from negligible to around 20% over the course of the experiment, suggesting a substantial movement of fluorescent lipids across the interface. Control experiments omitting ATP from the buffer showed minimal changes during the experimental timescale, with the percentage of fluorescence localized in the "acceptor" droplet not exceeding 0.03% over the course of 5 hours (see supplementary materials S2).

These results suggested that MsbA flippase activity had been recorded across an asymmetric DIB. For subsequent experiments, the hexadecane bath was upgraded into a machined PMMA dish, with channels to hold the DIB in place in a confined environment with a much smaller external volume. A microinjector was also added to the apparatus so that ATP could be injected into a pre-formed DIB to trigger flippase activity at a precise moment. When the experiment was repeated with these modifications, it became clear that our original assumption that the fluorescence changes of the droplets were due to flippase activity at the interface was overly simplistic. After injecting ATP into a single aqueous droplet of MsbA reconstituted into 95% DPhPC and 5% NBD labeled DOPE liposomes,

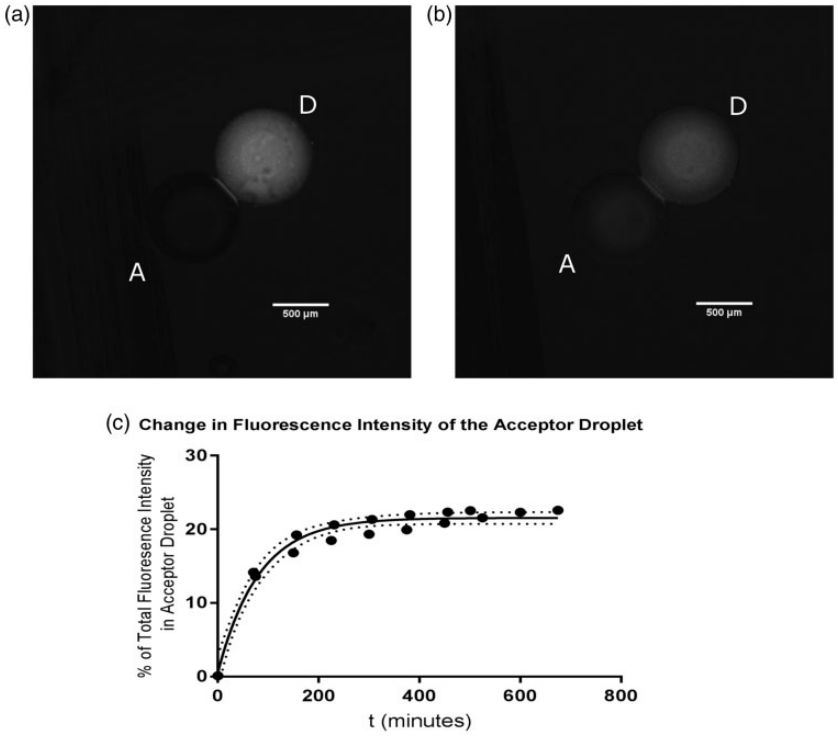

Figure 5. Panels a and b: Fluorescence microscopy image of a DIB composed of a donor droplet (D) containing MsbA reconstituted into 95% DPhPC liposomes labeled with fluorescent NBD-PE lipid (5%), interfaced with an acceptor droplet (A) containing DPhPC lipid only. Panel a was recorded at t = 0, panel b at t = 500 min. Panel c: A graph showing the fluorescent intensity changes of droplet A over time. The fluorescent intensity is displayed as a percentage of the total recorded fluorescent intensity across both droplets that was recorded in droplet A. Results from two experiments are shown along fitted with a one phase exponential decay curve (with 95% confidence bands).

fluorescence could be clearly recorded moving from the aqueous droplet into the surrounding non-polar hexadecane phase (Figure 6). The movement only occurred in the presence of both MsbA, ATP, and magnesium (Mg), demonstrating that active MsbA was facilitating the movement of either the NBD label or the entire labeled lipid from the droplet into the non-aqueous phase. This suggests that in the initial experiments, while MsbA activity was responsible for the increase in fluorescence in the acceptor droplets, it may have been mediated through the solvent instead of, or in addition to, flipping across the interface.

To discern whether the recorded fluorescence behavior corresponded to a phase transfer of fluorescent lipids or the detachment of the fluorescent label, an experiment was devised to allow the non-polar phase surrounding the droplet to be analyzed. First, 100 μL of a mix of reconstituted MsbA identical to the contents of the "donor" droplet was placed in a microcentrifuge tube. Then 100 μL of hexadecane was carefully floated on top of the aqueous layer. After 24 h, the hexadecane layer was siphoned off and analyzed for lipid content using thin layer chromatography (TLC) (Figure 7).

The results revealed that both NBD-labeled DOPE and DPhPC had transferred into the non-polar hexadecane layer. In the absence of MsbA, minimal lipid transfer occurred. The experiment was repeated with liposomes composed of DOPC/DOPE/DOPG and a mixture mimicking the composition of the *E. coli* inner membrane (DOPE/DOPG/cardiolipin). In each case, all of the lipids tested transferred to the non-polar phase in the presence of MsbA and ATP. Removing the ATP or including sodium orthovanadate in the buffer, which inhibits ATP hydrolysis by MsbA, significantly reduced the amount of lipid transferred.[70] The experiment was repeated without MsbA present initially, allowing time for a monolayer to form on the aqueous/non-polar interface. MsbA was then injected directly into the aqueous phase, both solubilized in detergent and reconstituted as liposomes. In both cases, lipid transfer to the non-polar phase was observed, though to a seemingly lesser magnitude than when MsbA was present from the very start of the experiment.

Taken together, these observations suggest MsbA activity is catalyzing the phase transfer of lipids from the aqueous to the non-polar phase. From these results, it is not possible to infer whether the MsbA is interacting directly with the lipids in the monolayer or if it is causing physicochemical changes in the liposomes which subsequently lead to monolayer disruption. It does suggest that MsbA may have far more lipid substrates than is currently thought, and that it may not be necessary for MsbA to span a lipid bilayer in order to be active. These observations also demonstrate that when using DIBs to study membrane protein function, protein interactions occurring away from the bilayer interface should be accounted for. The possibility of interactions between proteins and other chemical species within the lipid monolayer surrounding the droplet, and with the non-polar phase itself, may contribute to recorded results and must be given due consideration.

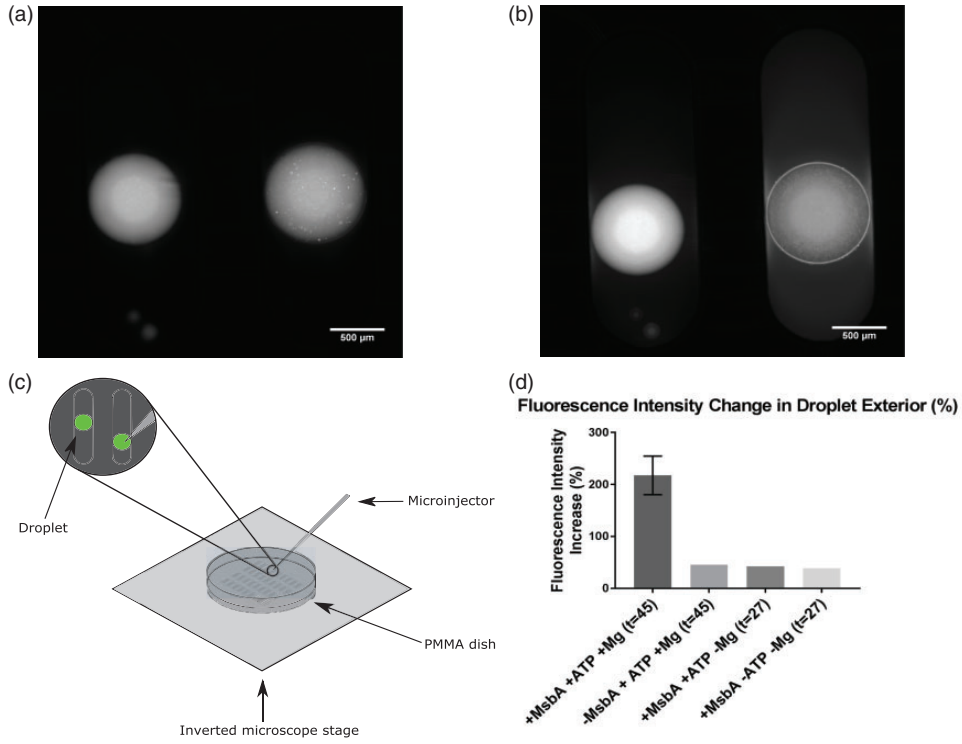

Figure 6. Panel a: Fluorescence microscopy image of two droplets, both composed of 95% DPhPC liposomes labeled with fluorescent NBD-PE (5%). The right hand droplet is comprised of proteoliposomes reconstituted with MsbA. Droplets are shown confined in shallow wells immediately after the injection of ATP via a micro-injection. Panel b: Fluorescence microscopy image of the same two droplets 110 min after ATP injection. Panel c: Schematic diagram of the microscope stage set up, with the view through the microscope objective shown as a cutaway. Panel d: Graph showing the percentage increase of the fluorescent intensity in the wells surrounding the droplets after ATP injection under a variety of experimental conditions. Time values are shown in minutes. (A color version of this figure is available in the online journal.)

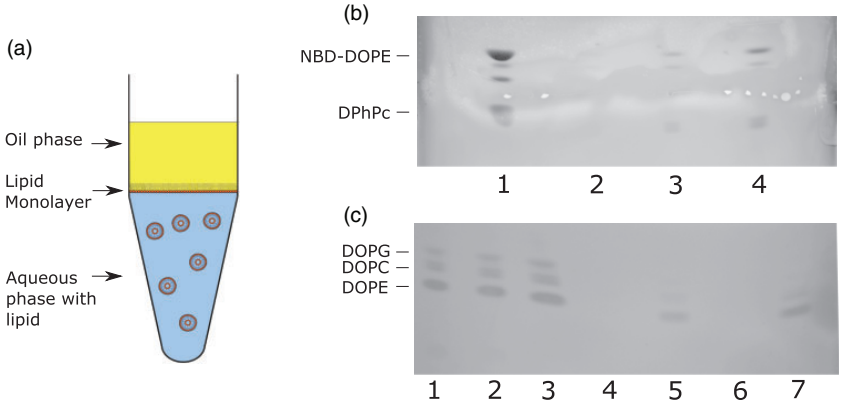

Figure 7. Panel a: Schematic diagram of the two-phase lipid transport experiment. Hexadecane phase is shown in yellow, aqueous phase in blue, and liposomes and lipid monolayer in orange. Panels b and c: Fluorescence images of thin layer chromatography plate analysis of the non-aqueous phase of a two-phase lipid transport experiment after 24 h. The mobile phase is a chloroform:methanol:water (65:25:4) mixture. In Panel b, lane 1 is 95% DPhPC 5% NBD-PE 5 μg standard and lanes 2, 3, and 4 are samples of the non-aqueous phase diluted 1:2 in chloroform under several conditions. 2: liposomes without MsbA, 3: No ATP or Mg, 1mM Na-orthovanadate present, 4: ATP and Mg present. In panel c, lanes are 1, 2, 3: DOPC (25%): DOPE (50%): DOPG (25%) 1 μg, 2 μg, and 3 μg standards in chloroform. Lanes 4, 5, and 6 are samples of the non-aqueous phase diluted in chloroform in the same manner as the previous panel. Lane 4: Liposomes without MsbA 5: No Mg present, 1 mM EDTA present, 6: No ATP present, 1 mM Na-orthovanadate present, 7: ATP and Mg present. (A color version of this figure is available in the online journal.)

Concluding remarks

DIBs are a versatile platform for studying a range of integral membrane protein properties. They offer many advantages over black lipid and Montal Mueller lipid bilayers, being significantly more stable and simpler to construct. Novel experiments can be designed using asymmetric bilayers and small aqueous volumes. DIBs have also demonstrated their utility for exploring the relationship between membrane protein function and lipid identity.

Some caution should be exercised when designing DIB-based experiments, as our own investigations show. Potential interactions with lipid monolayers and the surrounding solvent must be taken into account when designing and interpreting experiments. The identity of lipids

used to form DIBs must be considered carefully, bearing in mind their effect on membrane proteins function as well as the stability of the bilayer.

Compared to other artificial bilayers, DIBs are a relatively new technology and their full potential is far from realized. Despite this, their unique properties – robustness, simple assembly and versatility in reconstitution technique and lipid composition – would suggest that they are well placed to become an essential part of the membrane protein scientist's toolbox in the years to come.

Authors' contributions: MAB designed, carried out and analyzed the data from the MsbA study. All authors participated in the writing and editing of the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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